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Final Technical Report

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List of Acronyms

SERDP:	Strategic Environmental Research and Development Program
NSWC:	Naval Surface Warfare Center
RDX:	hexahydro-l,3,5-trinitro-1,3,5-triazine
HMX:	1,3,5,7-tetranitro-1,3,5,7-tetrazocine
FTIR:	Fourier Transform Infrared Spectroscopy
NMR:	Nuclear Magnetic Resonance Spectroscopy
EM:	Energetic Materials
CL-20:	2,4,6,8,10,12-hexaazaisowurtzitane
16S rDNA:	Subunit Ribosomal Deoxyribonucleic Acid
HPLC:	High Pressure Liquid Chromatography
LC:	Liquid Chromatography
GC:	Gas Chromatography
DSC:	Differential Scanning Calorimetry
MNX:	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
DNX:	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
TNX:	hexahydro-1,3,5-trinitroso-1,3,5-triazine
MDNA:	methylenedinitramine
BHNA:	bis-(hydroxymethyl)nitramine
NDAB:	4-nitro-2,4-diazabutanal
MDNA:	methylenedinitramine
ACS:	chemical meets the specifications of the American Chemical Society
TLC:	thin layer chromatography
IPTG:	isopropyl β-D-1-thiogalactopyranoside
PCR:	Polymerase Chain Reaction
xplA:	gene encoding a RDX-degrading cytochrome P450
BL21(DE3):	designation of competent strain
DMF:	dimethylformamide
SUL:	sulfanilamide
PBS:	phosphate buffered saline
LB:	Luria Broth
Kan:	kanamycin
rp-HPLC:	reverse phase HPLC
^{13}C-NMR:	Carbon-13 NMR

ACKNOWLEDGEMENT

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1. EXECUTIVE SUMMARY

In this SERDP proof-of-concept project, Infoscitex evaluated a possibility to generate explosives nitramines (RDX, HMX) using biosynthetic approaches. Nitramines are widely used by the U.S. military as key components of contemporary weapons. However, production of these nitro-energetics involves the use of highly concentrated hot nitric and sulfuric acids, results in the emission of nitrogen oxides, and is also a potential health hazard. The development of an alternative process for producing nitro-energetic compounds under milder conditions, in an environmentally friendly manner, and in higher yields would result in a significant saving and improvement of the quality of life in the surrounding communities.

The focus of this SERDP effort was to develop microorganisms capable of catalyzing bioconversion (oxidation-nitration) of the common RDX precursor, hexamine, into RDX in presence of nitrite and peroxide.

The program encompassed three main activities:

- Generation of a microbial strain that would be able to convert hexamine into RDX
- Generation of microbially produced RDX
- Characterization of the microbial product

The following outcomes were generated:

- A microbial strain deriving from a known microbial RDX degrader, *Rhodococcus* strain DN22, was engineered to exhibit elevated levels of cytochrome P450 expression and activity that would be required for hexamine to RDX conversion.
- The biomass of this microorganism was applied to attempt the hexamine to RDX conversion.
- The microbial product was analyzed for presence of RDX by FTIR, chromatography, and NMR spectroscopy.
- Formation of RDX was not detected. However, it is possible that some RDX-like intermediates were formed in trace amounts.

2. OBJECTIVE

The overall objective of the proposed effort was to develop an environmentally benign and economical microbial process for nitro-energetics production. The specific targets of this method of manufacture include:

- Elimination of hazardous or toxic liquid and solid byproducts
- Dramatic reduction of all waste streams
- Increase the quality and, potentially, yield of the energetic materials (EM)
- Exclude aggressive media (use of strong acids and harsh conditions) from the production process.

Overall, the Infoscitex team planned to achieve these goals by:

- Applying green microbiological synthesis
- Employing well-described and improved microorganisms containing biocatalytic machinery necessary for transformation of precursor materials to the targeted nitro-energetics
- Optimizing the process conditions, controls, and manufacturing technology

3. BACKGROUND

Infoscitex explored the feasibility of microbial production of nitro-based EM. As the processes and compounds of choice, RDX/HMX (nitramine) generation was selected. Microorganisms capable of withstanding high peroxynitrite concentrations are envisioned to include nitrite into the structure of water-insoluble EM. The grounds for the assumed approach are based on:

- Reported microbial activity in transformations of nitramines (1).
- Power of development of new microbial strains via directed microbial and enzyme evolution

3.1 Overview of Current Technologies for the Synthesis of Nitramines

Nitramines currently in use by the military include RDX and HMX. CL-20 (2,4,6,8,10,12-hexaazaisowurtzitane) was patented close to twenty years ago, yet is only beginning to find its

way into military stores. U.S. manufacture of RDX and HMX involves reaction of hexamine with nitric acid, ammonium nitrate, glacial acetic acid, and acetic anhydride. The reaction mixture is digested to destroy unstable byproducts, and air-borne byproducts, such as nitrogen oxides, sulfur oxides, and acid mists, must also be treated. Waste-water treatment sludge resulting from the manufacture of nitramines is classified as hazardous waste and is subject to EPA regulations. Wastes containing RDX have been incinerated by grinding the explosive wastes with a flying knife cutter and spraying the ground material with water to form slurry. Rotary kiln or fluidized bed incineration methods are acceptable disposal methods for HMX-containing wastes. The primary disadvantage of open burning or incineration is that explosive contaminants are often released into the air, water, and soils.

CL-20 synthesis requires major technological advances to become economical. The final stage of its synthesis involves nitration of the acetylated and benzylated amino group (Figure 1).

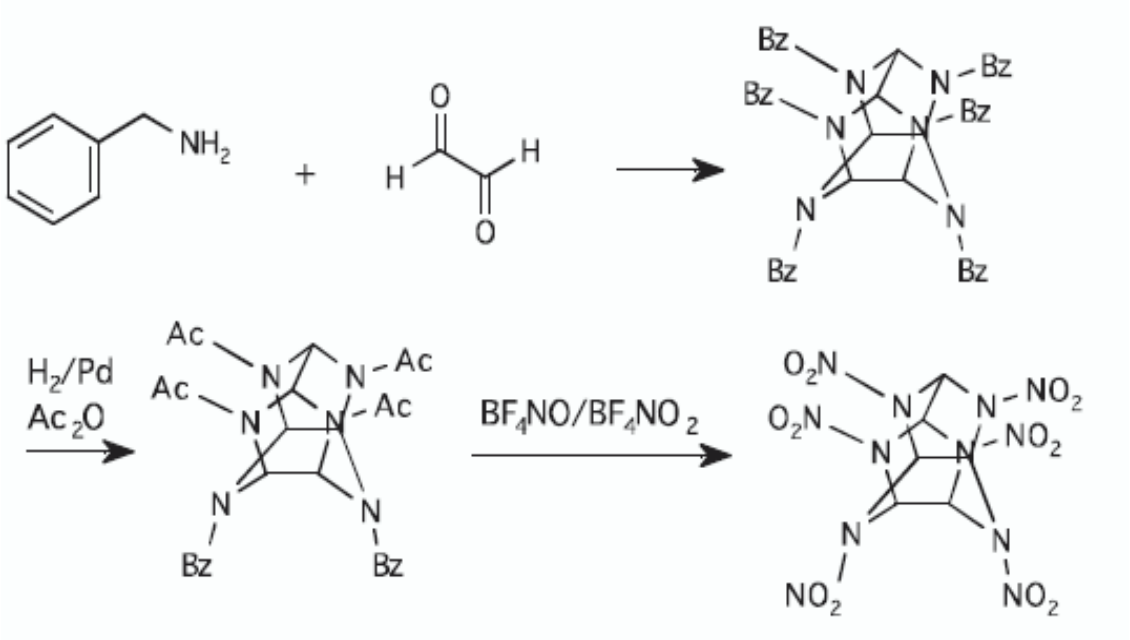


Figure 1 CL-20 Synthesis

3.2 Microbiological Synthesis: Opportunity for Nitramine Production

Current synthetic methods for the production of RDX and HMX utilize hexamine as the precursor. Hexamine is an industrial chemical available on a large-scale. In bio-production, it

would be continued to be used as the precursor. The schematics of potential synthesis of nitramines by microorganisms are depicted in Figure 2.

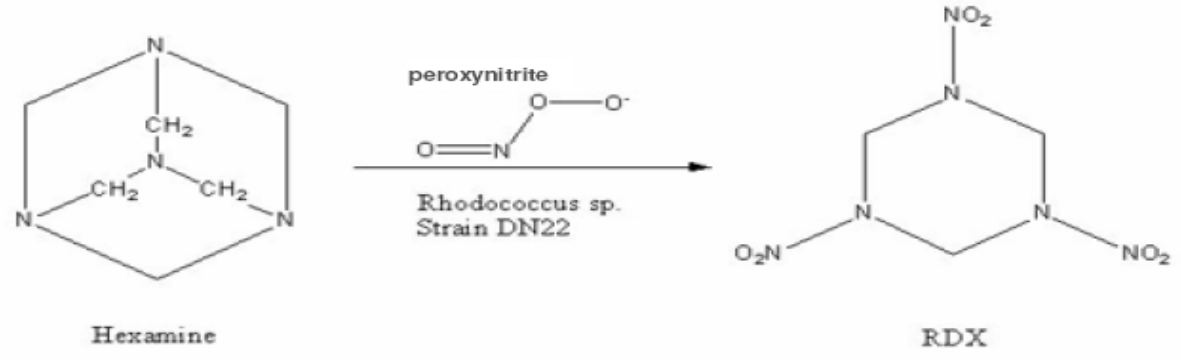


Figure 2 Proposed scheme for microbial production of RDX from Hexamine

The isomeric composition of the target EM will be controlled by adjusting or optimizing the conditions of fermentation. No need will exist for strong acids and harsh conditions to nitrate the precursors. Tight control over product composition will alleviate a significant problem with U.S. production, as it has been determined that HMX contamination of RDX may be responsible for its dangerous sensitivity.

CL-20 CL-20 is a promising high-density, high-power EM, but its industrial manufacture is hampered by its prohibitive cost. In the feasibility proof-of -principle stage of the proposed program CL-20 synthesis will not be addressed; however, once the general approach of microbial nitration of the EM precursors is demonstrated, generation of a strain capable of same reaction for CL-20 will be attempted. This work will be facilitated by the recent advances in isolation and characterization of the strains capable of CL-20 de-nitrification under anaerobic conditions. The CL-20 transforming bacteria have been isolated in pure cultures and characterized in terms of cell morphology, physiology and taxonomic position by sequencing of 16S rDNA. The microorganisms bear the enzymes capable of anaerobic cleavage of the N-NO_2 bond (2).

3.3 Approach

Nitroarenes and nitramines are widely used by the U.S. military as key components of contemporary weapons. However, production of these nitro-energetics involves the use of highly concentrated hot nitric and sulfuric acids, results in the emission of nitrogen oxides, and is also a

potential health hazard. The development of an alternative process for producing nitro-energetic compounds under milder conditions, in an environmentally friendly manner, and in higher yields would be a vast economic saving and improve the quality of life in the surrounding communities. The objective of the proposed program is to develop a biological process through which military-grade EM can be produced without generating hazardous wastes, and with minimal generation of hazardous or toxic by-products.

The new technological process would include several major stages, including microbial production, isolation and purification, and quality control. Usage of aggressive, concentrated inorganic acids will be eliminated, while the need for organic solvents will be greatly reduced. Improved microorganisms will be produced for the synthesis of targeted compounds. The microbial process will essentially be a conversion of the precursors of the energetic ingredients into their nitrated forms (which are water insoluble) in water-based medium in a bioreactor supplied with peroxynitrite as a nitration agent. The driving force of the microbial nitration will be the need for microorganisms to detoxify their habitats from peroxynitrite by reacting it with precursors of EM. In a large scale program the capability of the microorganisms to perform such a nitration will be evolved by mutagenesis and exposure to selective pressure in the form of elevated concentration of peroxynitrite. This process can also be considered as biocatalytic nitration of EM precursors with microorganisms serving as the catalyst. The process will employ evolutionary-established, highly regioselective microorganisms. Thus, downstream processing will be facilitated due to lower isomeric diversity of the microbially-produced nitro-energetics. Generation of the EM in microbial cultures, as well as some initial insights into the product isomeric purity, will be confirmed by analysis (high pressure liquid chromatography, HPLC, liquid chromatography, LC, gas chromatography, GC, differential scanning calorimetry, DSC, nuclear magnetic resonance (NMR) spectroscopy and/or similar methods). This approach has a strong scientific basis; however, technical implementation may present difficulty. Therefore, an initial program aimed at concept demonstration was performed.

It is anticipated that the reduction of a successful microbial technology of nitramine production to practice will have many beneficial impacts on Department of Defense end-users. Most significant of these are: 1) environmental burdens, along with associated costs, will be reduced significantly, and 2) energetic product quality, and potentially yield, will be improved.

3.4 Prior Work by the Research Community

The idea of applying growing knowledge of explosives biotransformation and/or biodegradation pathways in living organisms is rather new. Apart from the subject program, the earliest mentioning of such an approach was published by a researcher at the U.S. Army Engineer Research and Development Center, Vicksburg, MS (3). The authors provide an excellent review of pathways for microbial degrading of nitramines. Figure 3 depicts several processes relevant to biodegradation of RDX (3).

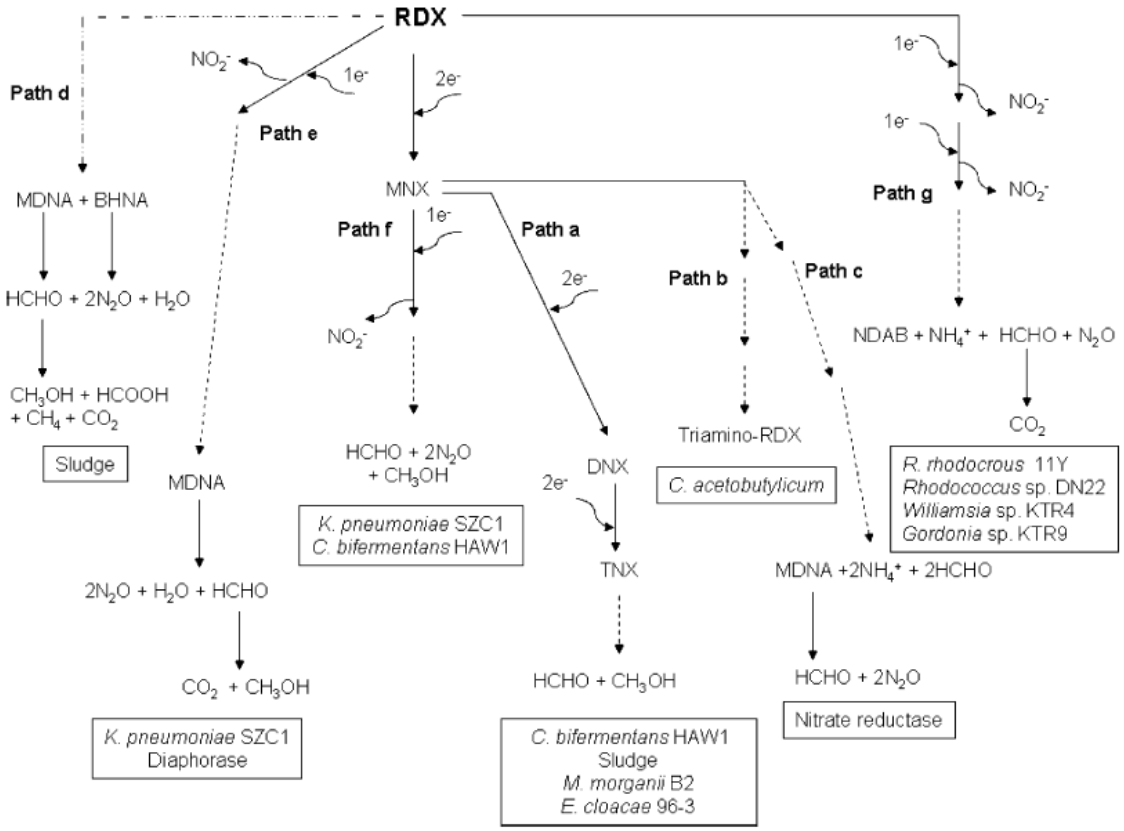


Figure 3 RDX microbial degradation (3)

Abbreviations:
RDX, hexahydro-1,3,5-trinitro-1,3,5- triazine,
MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
DNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine,
MDNA, methylenedinitramine,
BHNA, bis-(hydroxymethyl)nitramine
NDAB, 4-nitro-2,4-diazabutanal
MDNA, methylenedinitramine

As it can be seen in the Figure 3, while the overall degradation process is multi-stage and requires multi-enzymatic reactions, the first stages of microbial degradation pathways include nitrite ion release from the RDX molecule. Thus, one step bioconversion of hexamine into RDX via oxidative nitration (Figure 2) was selected as the aim of the study. Certainly, the question if the degradation reaction could be reversed and used to synthesize the target product remains to be answered.

4. MATERIALS AND METHODS

Microbial strains and culture conditions. *Rhodococcus* strain DN22 was a gift of Prof. Jim Spain, (School of Civil and Environmental Engineering at the Georgia Institute of Technology). The strain was maintained in LB medium. The strain mutants were selected and maintained in a kanamycin supplied medium as specified in the text.

Solvents and reagents. The ACS grade solvents and reagents were purchased from Sigma-Aldrich (St. Lois, MO), unless specified differently.

FTIR spectrometry. The FTIR spectrometry was performed using Shimadzu IR Prestige Spectrometer (Shimadzu Scientific Instruments, Columbia, MD)

NMR Spectroscopy. The NMR spectroscopy was performed using Bruker DRX-500 MHz NMR spectrometer (Bruker BioSpin Corp. Billerica, MA)

HPLC Chromatography. The HPLC chromatography was performed on the Agilent Technologies 1100 Series HPLC station (Agilent Technologies, Andover, MA) equipped with WATERS SPHERISORB C8 COLUMN 250x4.6mm 10um column (Waters Corp, Milford, MA). The sample elution was isocratic. HPLC grade methanol was used as the eluent. The peaks were detected at 220 and 250 nm. The 99 % pure RDX from Accustandard (New Haven, CT) served as a reference standard.

Preparative TLC. The preparative thin layer chromatography was performed using a Silica Gel G 1000µm 10x20cm plate. The HPLC grade methanol was the eluent. The 99 % pure RDX from Accustandard (New Haven, CT) served as a reference standard. Upon completion of the TLC run the spots were identified with Iodine vapor staining.

Cloning the P-450 enzyme from Rhodococcus strain DN22. Genomic DNA was isolated from Rhodococcus strain DN22 and the *xplA* gene (encoding the RDX-degrading P-450 enzyme) was amplified using primers R.xplA.f.pRP1B (5'-GTGGAGCATATGACCGACGTAACTGTCC-3') and R.xplA.r.pRP1b (3'GGCTTTTGTCGGCTAGCAGGACAGGACTTCGAACCAAGA 5'). Plasmid DNA was isolated and the correct plasmid pRP1B-xplA.DN22 was confirmed by digesting with the restriction enzymes EcoRI, NdeI and Hind III. The plasmid was then sequenced using both the forward and reverse primers used for cloning above. Sequencing result showed that xplA from strain DN22 was identical to that in strain 11Y.

Detection of nitrite from bacterial degradation of RDX. 10 mL LB/Kan100 cultures were started from 1-day single colonies of (1 colony for each strain) and grown at 37°C 250rpm overnight. Overnight cultures were diluted 100 fold (start A600~0.05) into 50 mL LB/Kan100 with 0.5 mM $FeCl_3$, 5 μg/L riboflavin, and 1 mM 5-ALA (aminolevulinic acid) (4) with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) or no IPTG. Cells were grown at 30°C 250rpm until A600~1.2-1.4, then cells were harvested by centrifugation at 25°C. Cells were resuspended in 0.1 M sodium phosphate buffer pH 6.5 and dispensed into glass vials. RDX (1/1000 (v/v) dilution of 1000x stock in DMF) was added and contacted with cells at 30°C 250rpm in sealed glass vials. At various time points, 1 mL cell culture was removed and cells were pelleted by centrifugation. 0.8 mL supernatant was transferred to a fresh microcentrifuge tube and 160 μL SUL was added and mixed. After 5 min at room temperature, 160 μL NAD was added and mixed. A543 was measured after 15 min at room temperature. For nitrite concentration vs. time plots, the nitrite concentration was calculated using a standard curve (A543 = 0.0391[NO2-] (μM)).

Error-prone PCR to create beneficial mutations. Error-prone PCR was conducted to generate random mutations in xplA, then the DNA was ligated into pRP1B. Colonies were screened in 96 well plates by detecting nitrite spectrophotometrically. 200 μL per well with LB Kan 100 μg/mL were used to culture 1-day single colonies of error-prone PCR BL21(DE3)/pRP1B-xplA.DN22 (BL21(DE3)/pRP1B was used as a negative control, 5 replicates, along with wild-type BL21(DE3)/pRP1B-xplA.DN22 as the positive control). These cultures were grown at 37°C at 250rpm overnight. The overnight cultures were diluted 100 fold (start A600~0.05) into 200 μL/well LB Kan 100 with 0.5 mM $FeCl_3$, 5 μg/L riboflavin, and 1 mM 5-ALA (aminolevulinic acid) (4) and 0.1 mM IPTG was added. Cells were grown at 30°C 250 rpm for ~6hrs, then 100

μM RDX (4 μL 5mM stock in DMF) was added to each well and contacted with cells for 2 hrs at 30°C 250rpm. The absorbance at 600 nm was then measured using a Tecan Sunrise absorbance plate reader. For the nitrite assay, 20 μL SUL was added to each well and allowed to stand for 5 min at room temperature; then 20 μL NAD was added to each well. After 15 min at room temperature, the absorbance at 543 nm was taken using the Tecan Sunrise plate reader.

Evaluation of possible RDX production.

Baseline reaction with no biological catalyst. All the reagents were of ACS grade and were purchased in Sigma-Aldrich. Diionized water was produced in house in a reverse osmosis unit (18.2 M Ω). 100 mL of 0.1 M solution of $NaNO_2$ in 0.1 M phosphate buffered saline pH 7.4 (PBS) was mixed with 100 mL of 3% H_2O_2 in an ice bath in one liter beaker. To this solutions 100 mL of a 1, 3, 5 and 10 % solution of hexamine in water containing 0.001 M $FeCl_3$. The mixtures were gently stirred with a glass rod for 60 min. During mixing 20-mL aliquots were collected every 5 min. The aliquots were dried to the constant mass at room temperature under flow of dry air, and eventually under vacuum. The solid residues were extracted with 3 mL acetone for 72 hrs. The acetone extracts were centrifuged to remove residual solids. Samples produced in the absence of *Rhodococcus* cells were analyzed using FTIR.

*Baseline reaction with unimproved biological catalyst***.** The microorganism biomass was grown in LB broth in culture flasks and harvested by centrifugation. 50 g of wet biomass was collected and used in the test of RDX formation in a manner similar to the procedure described above (A. Baseline reaction with no biological catalyst). In each of the aliquots containing RDX raw materials and peroxide 2 g of wet biomass of the *Rhodococcus* DN22 was added and suspended. The microbial suspensions were incubated at 37 °C for 24 hrs, dried, and extracted with acetone as described above.

Reaction with improved biological catalyst. The improved microorganism, designated as BL21(DE3)/pRP1B-xplA.DN22, showing highest nitrite production rate was grown in LB medium supplied with 100 mg/L kanamycin (LB/Kan 100) under conditions optimized for the unimproved control. More specifically, 2 g of wet biomass was mixed with 100 mL of 0.1 M solution of $NaNO_2$ in 0.1 M phosphate buffered saline pH 7.4 (PBS) containing 100 μm IPTG and 100 mL of 3% H_2O_2 in an ice bath in one liter beaker. To this solutions 100 mL of a 1 %

solution of hexamine in water containing 0.001 M $FeCl_3$. The suspension was incubated at 37 °C for 24 hrs, dried, and extracted with acetone as described above.

5. RESULTS AND ACCOMPLISHMENTS

5.1. Strain Improvement

5.1.1 Cloning the RDX-degrading enzyme. To enable mutagenesis of the P-450 enzyme from *Rhodococcus* strain DN22, we first cloned the enzyme into E. coli using the pRP1B vector as shown in Figure 4. This strategy includes the addition of a 20 amino acid histidine tag so that the enzyme may be easily isolated. Sequencing shows that the correct gene was cloned.

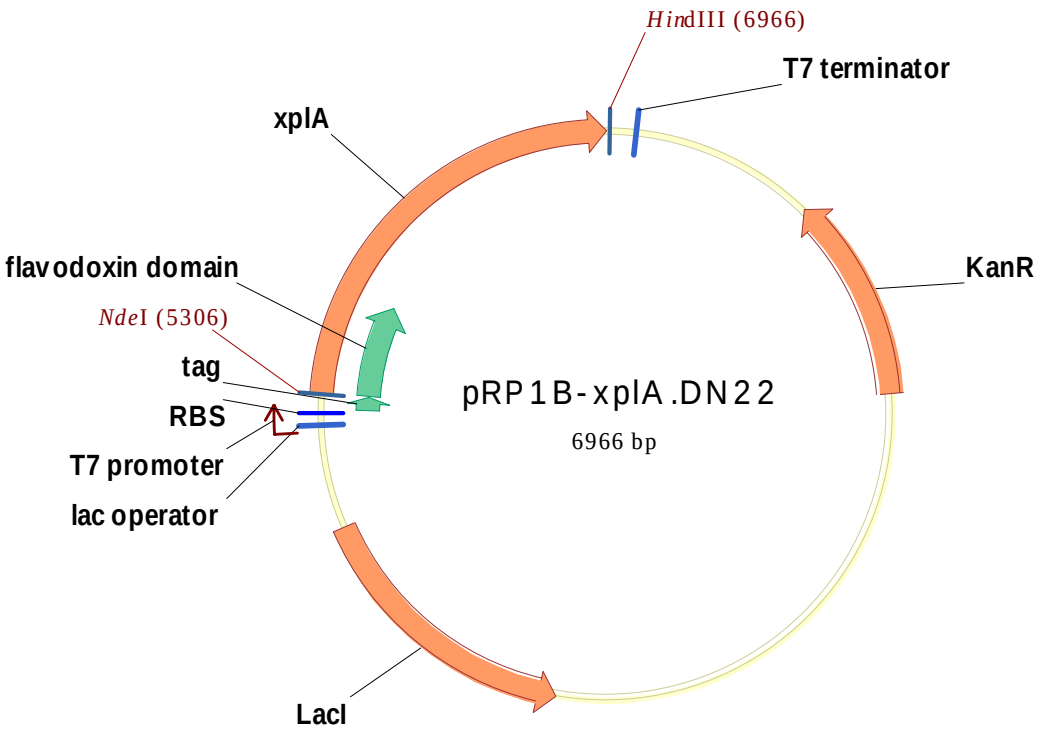


Figure 4 ***E. coli*** **cloning vector for expressing the P-450 enzyme (XlpA) from** ***Rhodococcus*** **strain DN22**.

5.1.2 Detection of nitrite from bacterial degradation of RDX.

Addition of IPTG to induce expression of the RDX-degrading P-450 enzyme from *Rhodococcus* strain DN22 (when expressed in *E. coli*) was necessary to detect nitrite from the degradation of RDX. Figure 5 shows roughly 60 µM nitrite formed from 50 µM RDX in 30 min. Therefore, the

E. coli strain has high activity for degrading RDX (there was no nitrite formed from the same cells that lack the P-450 enzyme). The optimum concentration of IPTG was found to be 100 µM. Similar results were found for RDX from 4.5 to 50 µM..

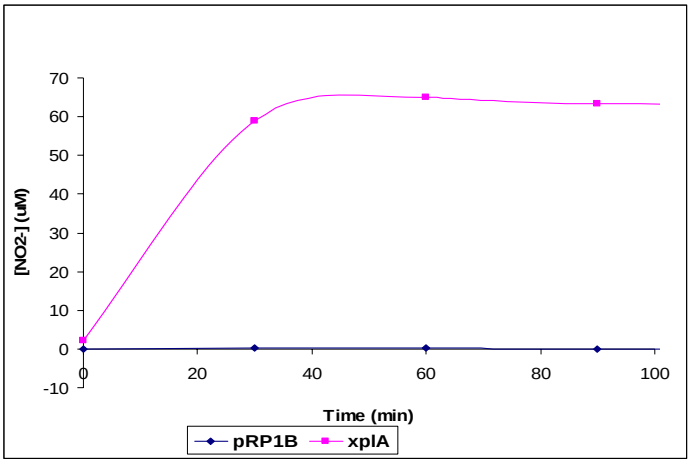


Figure 5 RDX-degrading activity of the whole cells of BL21(DE3) expressing *xplA* (pRP1B is the empty vector as negative control).

5.1.3 Detection of nitrite from error-prone PCR mutants degradation of RDX. Random mutations were introduced into the gene that encodes the P-450 activity (xlpA) via error-prone polymerase chain reaction so that new protein variants could be created that would have enhanced RDX degradation. These new XlpA variants were identified by assaying for enhanced nitrite formation from RDX. One mutant, G21, was identified that has slightly higher activity (Figure 6). We sequenced several mutants and found the PCR error rate was 0.2 to 0.6%.

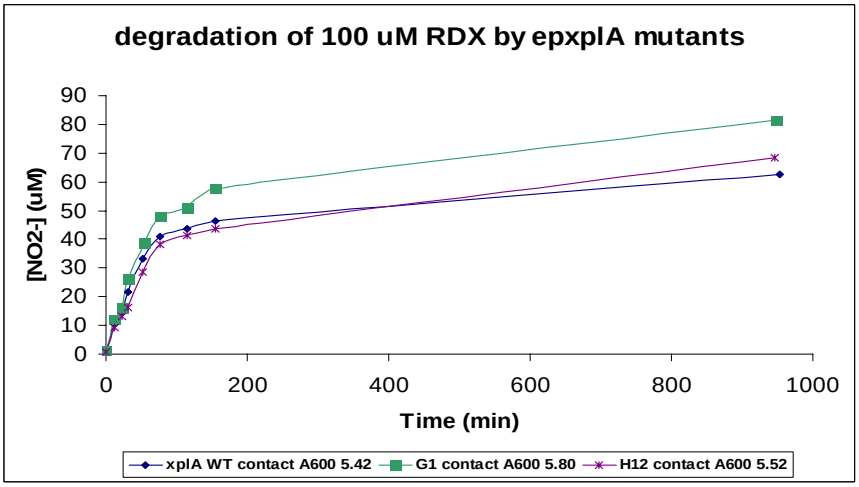


Figure 6 RDX degradation by wild type XplA and mutants G1 and H12.

5.2. Evaluation of the RDX formation

5.2.1 Baseline reaction with no biological catalyst.

During the reaction none of the experimental reaction mixtures showed formation of precipitates that could be attributed to formation of RDX. Samples produced in the absence of cells were analyzed using FTIR. The spectra of samples of reaction mixtures containing maximum hexamine concentration (10%) after 5, 20, and 60 minutes of reaction show some absorption at 1650-1700 and 1250-1300 cm^{-1}, which are characteristic group absorptions for nitramines and amines (Figure 7). The 1250 to 1300 cm^{-1} region was overcrowded and could not be used to judge reaction progress. However, the relative peak areas at 1650 – 1700 cm^{-1} showed some increase during the 60 minute reaction. This result is an indicator of potential presence of nitramines.

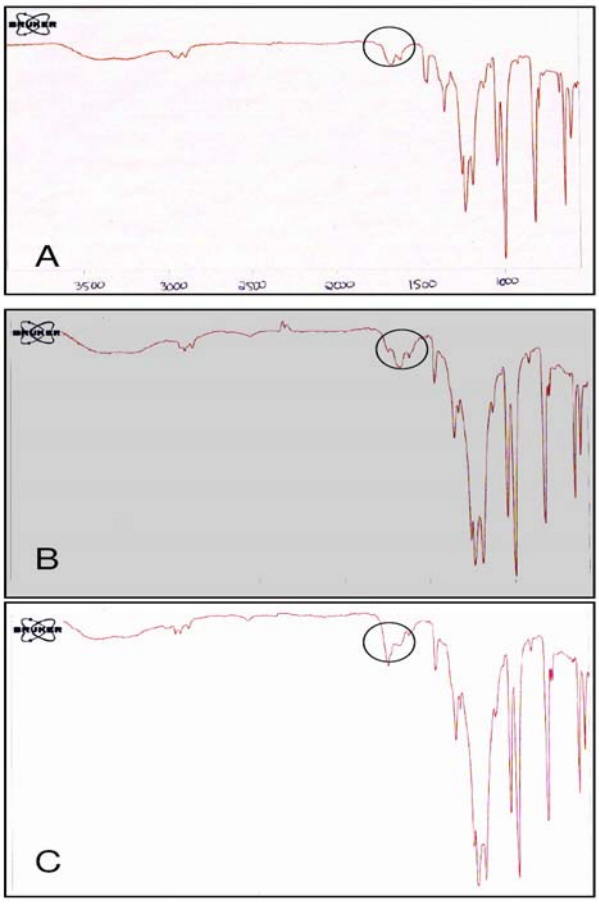


Figure 7 IR Spectra of the reaction mixtures containing no biocatalyst at 5, 30, and 60 min of reaction (A, B, and C respectively). The 1650-1700 cm^{-1} region is circled.

5.2.2 Baseline reaction with unimproved biological catalyst.

The spectra of samples of reaction mixtures (Figure 8) containing 1,3, and 10 % hexamine concentration after 24 hr reaction at 37 °C showed some absorbance at 1650-1700 and 1250-1300 cm^{-1}, which are characteristic group absorptions for nitramines and amines. The 1250 to 1300 cm^{-1} region was overcrowded and could not be used to judge reaction progress. However, the relative peak areas at 1650 – 1700 cm^{-1} were increased for the reaction mixture containing 1 % hexamine. The 3 and 10 % hexamine mixture exhibited the peak relative areas similar to reaction containing no biocatalyst. This result is an indicator of potential generation of nitramines. The observed decrease of the characteristic peak areas for 3, and 10 % hexamine compared to 1 % hexamine may be explained by increase of inhibiting (toxic) effects of media containing elevated concentrations of hexamine.

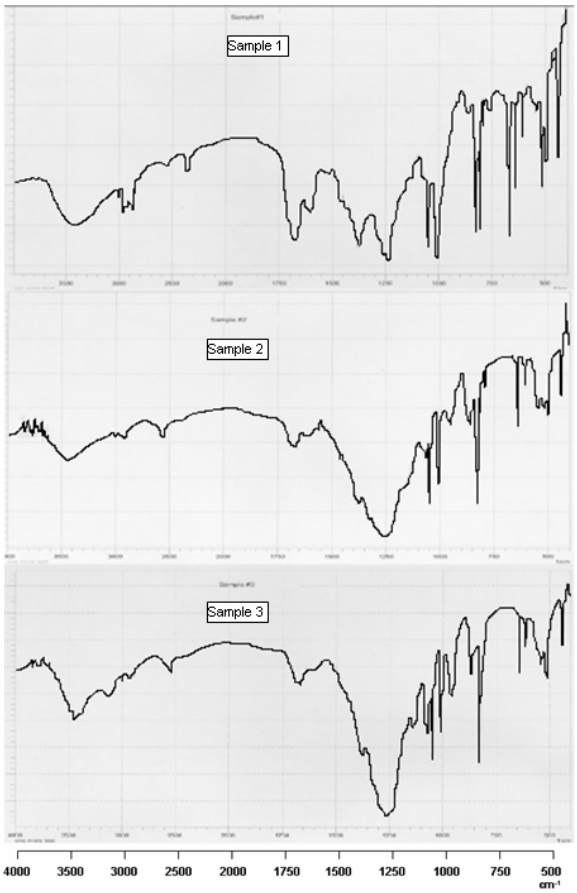


Figure 8 IR Spectra of the reaction mixtures containing biocatalyst and 1, 3, and 10 % hexamine (Sample 1, 2, and 3 respectively).

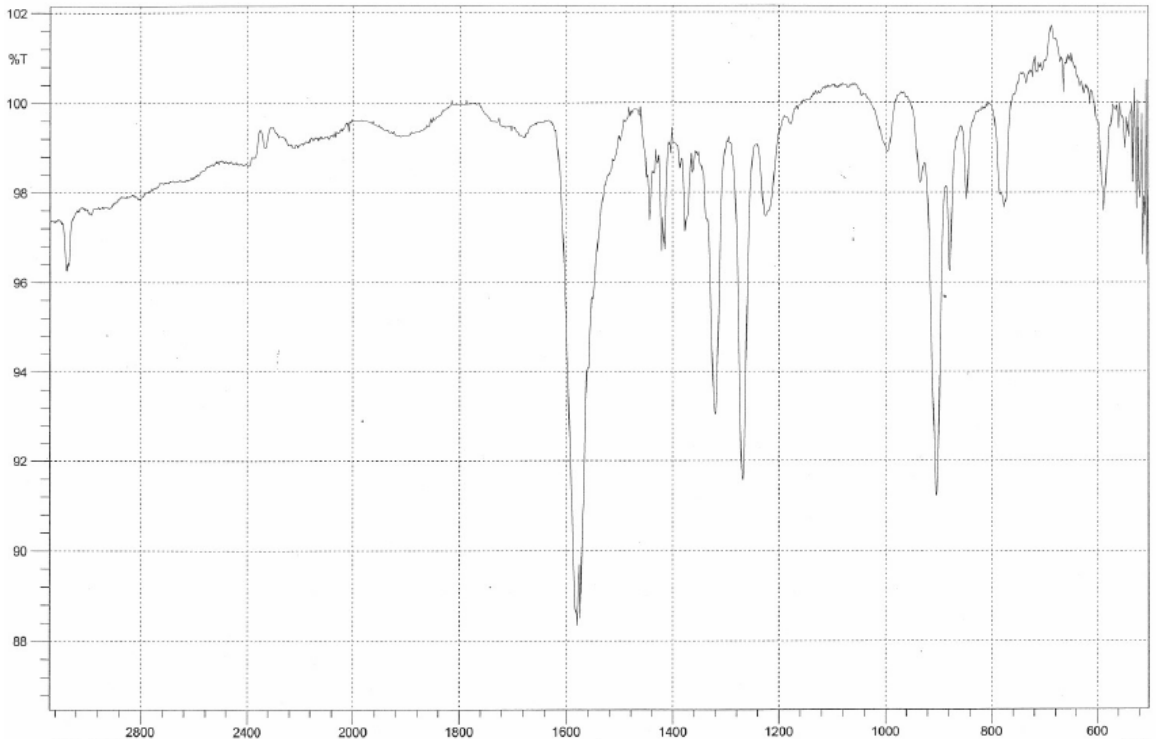
5.2.3 Reaction with improved biological catalyst.

After the reaction was carried out as described in the Materials and Methods section total of 15 mL of the acetone extract of the reaction mixture was obtained. The extract was dried over 1.0 g of dry magnesium sulfate during 72 hours. The extract was centrifuged. The volume of the acetone supernatant was reduced to approximately 1.5 mL under dried under flow of nitrogen gas. The concentrate was centrifuged and 1.0 mL of the clear concentrated supernatant was obtained. Its analysis by FTIR and HPLC revealed a rather complex structure of the acetone extract. Thus, the FTIR spectra (Figure 9) showed that the microbial product apparently exhibited an distinct absorbance peak at the 1650 – 1700 cm^{-1} (amine region), while the absorbance in 1250-1300 cm^{-1} region was very small (also amine region).

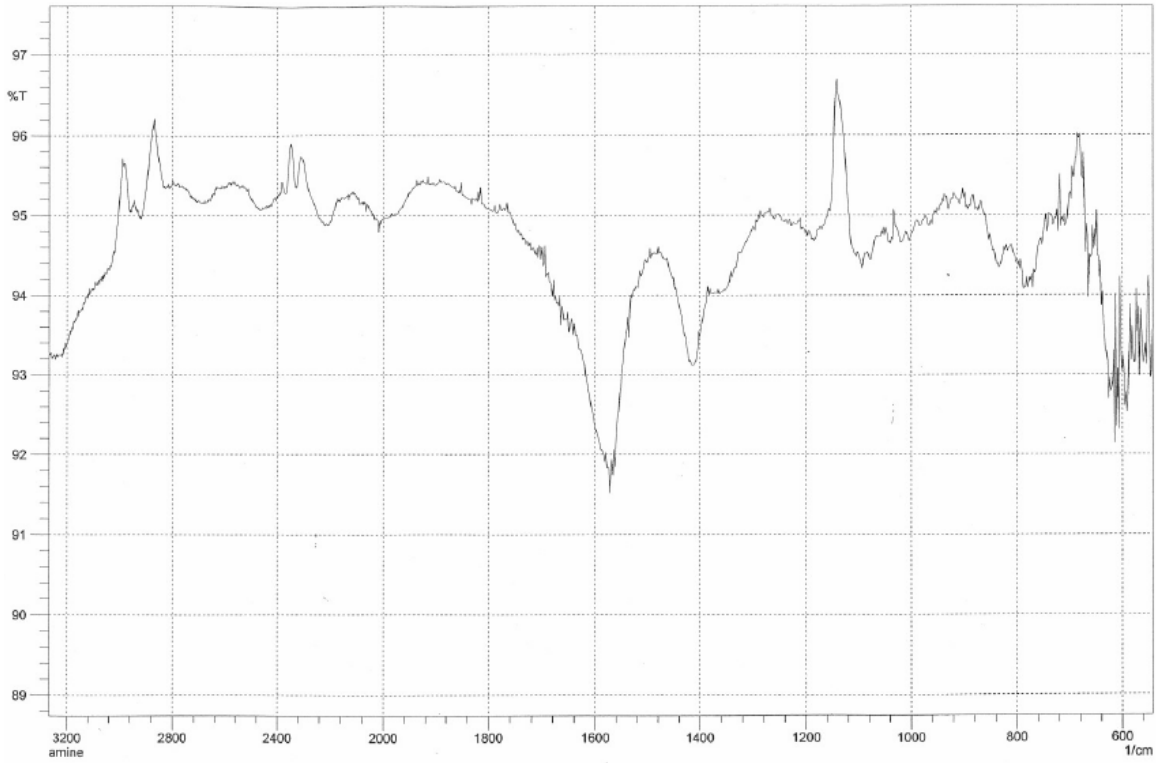
Further characterization of the biocatalytically prepared product by rp-HPLC (Figure 10 A and B) confirmed a complex composition of the culture extract, while suggesting presence of minute amounts (0.011 %) of a compound eluted very closely to the RDX standard (retention time 16.260 min and 16.291 min for the RDX standard and microbial product respectively, Figure 10 A&B).

5.2.4 Isolation of bioconversion product. In order to further characterize the microbial product an attempt was undertaken to collect, concentrate, and analyze the eluted closely to RDX compound by preparative TLC.

The acetone extract was applied as a narrow strip on the TLC starting line and dried by flow of nitrogen gas. After the chromatogram elution in methanol the plate was dried in a flow of dry nitrogen gas. The location of RDX standard spot was determined by cutting the standard RDX lane from the TLC plate and placing it into an iodine vapor chamber; so that the position of the RDX spot was revealed. From the remaining portion of the plate the adsorbent in the area corresponding to location of the RDX spot (1.90 g) was scraped and extracted successively with three changes (total 20 mL) of the HPLC grade methanol. The extract was centrifuged. The collected supernatant was dried overnight over 3 g of dry magnesium sulfate, centrifuged, collected and dried under flow of the nitrogen until constant weight. The obtained residue (7.3 mg) was evaluated using NMR.



A



B

Figure 9 FTIR spectra of the RDX standard (A) and product of biocatalytic reaction (B) (transmittance as function of the wavenumber).

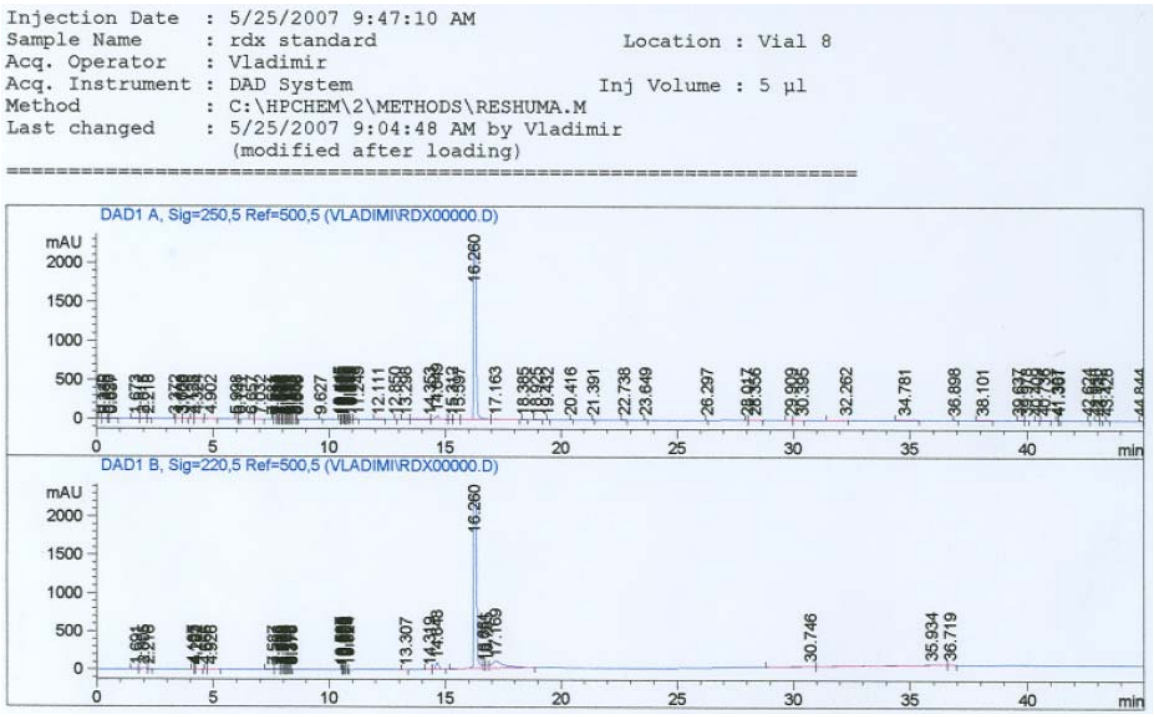


Figure 10 A rp-HPLC chromatogram of the RDX standard.

Note the RDX standard retention time at 16.260 min

The NMR data are presented in Figure 11 and 12 A and B. As long as both hexamine and RDX structures are symmetrical and, therefore, only one shift line would be observed in the spectra of these molecules, it was important to verify the existence of differences in chemical shifts in the spectra of the RDX standard, hexamine, and the microbial product. Figure 11 shows a spectrum of the raw material, hexamine. As it follows from the spectrum the ^{13}C chemical shift of hexamine is 72.0 ppm.

```
Injection Date  : 5/22/2007 6:57:58 PM          Seq. Line :   1
Sample Name     : RDX sample                    Location : Vial 9
Acq. Operator   : Vladimir                         Inj :   5
Acq. Instrument : DAD System                  Inj Volume : 50 µl
Sequence File   : C:\HPCHEM\2\SEQUENCE\RESHMA.S
Method          : C:\HPCHEM\2\METHODS\RESHUMA.M
Last changed    : 5/22/2007 1:04:09 PM by Reshma
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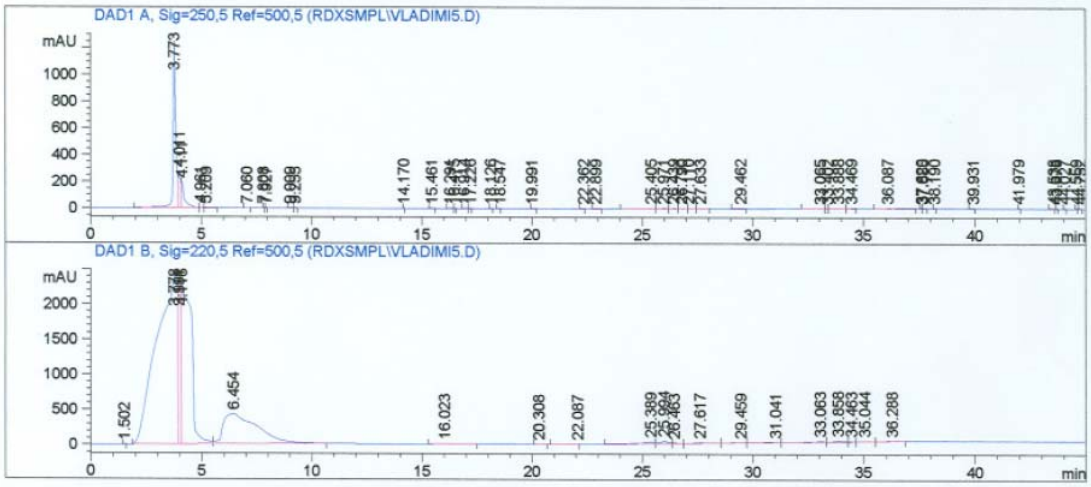



```
================================================================================
                              Area Percent Report
================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=250,5 Ref=500,5

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   3.773 PV    0.1158 1.04749e4  1249.48877  70.4830
   2   4.011 VV    0.0983 1914.14343  271.32657  12.8798
   3   4.111 VV    0.1390 1950.13782  191.24564  13.1220
   4   4.961 VB    0.0643   11.41895    2.36646   0.0768
   5   5.209 BP    0.2028    4.29102 2.54408e-1   0.0289
   6   7.060 BB    0.1639    6.04813 4.46840e-1   0.0407
   7   7.808 BB    0.0329 2.34443e-1 1.01748e-1 1.578e-3
   8   7.927 PB    0.0308 2.42629e-1 1.14120e-1 1.633e-3
   9   9.009 PB    0.0000 4.62780e-2 4.71372e-2 3.114e-4
  10   9.255 BB    0.0686 7.44130e-1 1.43355e-1 5.007e-3
  11  14.170 PB    0.0230 8.59770e-2 6.67107e-2 5.785e-4
  12  15.461 BP    0.1013 6.61604e-1 8.27465e-2 4.452e-3
  13  16.291 BP    0.1370    1.69175 1.50642e-1   0.0114
  14  16.475 VB    0.0460 3.54745e-1 1.08883e-1 2.387e-3
  15  16.914 PP    0.1190    1.30013 1.34240e-1 8.748e-3
  16  17.226 VV    0.0854 6.00779e-1 9.82115e-2 4.042e-3
  17  18.126 BB    0.0340 1.63564e-1 6.81392e-2 1.101e-3
  18  18.547 BV    0.0679 6.24190e-1 1.17704e-1 4.200e-3

DAD System 5/22/2007 7:43:05 PM Vladimir                    Page 1 of 3
```

Figure 10 B rp-HPLC chromatogram of the microbial product.

Note a peak number 13 of retention time of 16.291 min in the Peak Table.

```
Injection Date  : 5/22/2007 6:57:58 PM          Seq. Line :   1
Sample Name     : RDX sample                    Location : Vial 9
Acq. Operator   : Vladimir                          Inj :   5
Acq. Instrument : DAD System                  Inj Volume : 50 µl
Sequence File   : C:\HPCHEM\2\SEQUENCE\RESHMA.S
Method          : C:\HPCHEM\2\METHODS\RESHUMA.M
Last changed    : 5/22/2007 1:04:09 PM by Reshma
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
19	19.991	BP	0.0000	1.06618e-1	4.83193e-2	7.174e-4
20	22.362	BP	0.1151	8.71784e-1	9.67397e-2	5.866e-3
21	22.899	BB	0.1138	7.24291e-1	7.83833e-2	4.874e-3
22	25.405	PV	0.4680	66.24158	1.67293	0.4457
23	25.971	VV	0.3169	74.87852	2.96241	0.5038
24	26.439	VV	0.2587	54.33252	2.50795	0.3656
25	26.790	VV	0.2646	47.11055	2.14196	0.3170
26	27.110	VB	0.1935	24.19664	1.53811	0.1628
27	27.633	BV	0.2615	25.05489	1.19061	0.1686
28	29.462	BB	0.1237	5.17933	5.13365e-1	0.0349
29	33.065	BV	0.3127	13.06195	4.96811e-1	0.0879
30	33.402	VV	0.1031	3.11663	3.82491e-1	0.0210
31	33.888	VV	0.3201	39.38258	1.49147	0.2650
32	34.469	VV	0.2530	16.30564	7.76561e-1	0.1097
33	36.087	PB	0.7548	116.32918	1.81255	0.7827
34	37.620	BV	0.0787	2.01249	3.41641e-1	0.0135
35	37.685	VB	0.1113	2.74181	3.09788e-1	0.0184
36	38.190	BP	0.0443	2.27081e-1	7.72047e-2	1.528e-3
37	39.931	PP	0.0000	2.79427e-2	4.11694e-2	1.880e-4
38	41.979	BP	0.0262	1.39661e-1	8.96099e-2	9.397e-4
39	43.538	BP	0.0802	6.38281e-1	1.00407e-1	4.295e-3
40	43.678	BB	0.0375	2.49992e-1	9.87210e-2	1.682e-3
41	44.027	BP	0.0372	1.66261e-1	7.12947e-2	1.119e-3
42	44.559	BB	0.0363	2.87396e-1	1.10528e-1	1.934e-3
43	44.752	BP	0.0744	5.30044e-1	9.30896e-2	3.567e-3
Totals :				1.48616e4	1735.40641	

```
 Results obtained with enhanced integrator!
```

Figure 10 B Chromatogram of the microbial product (Continued)

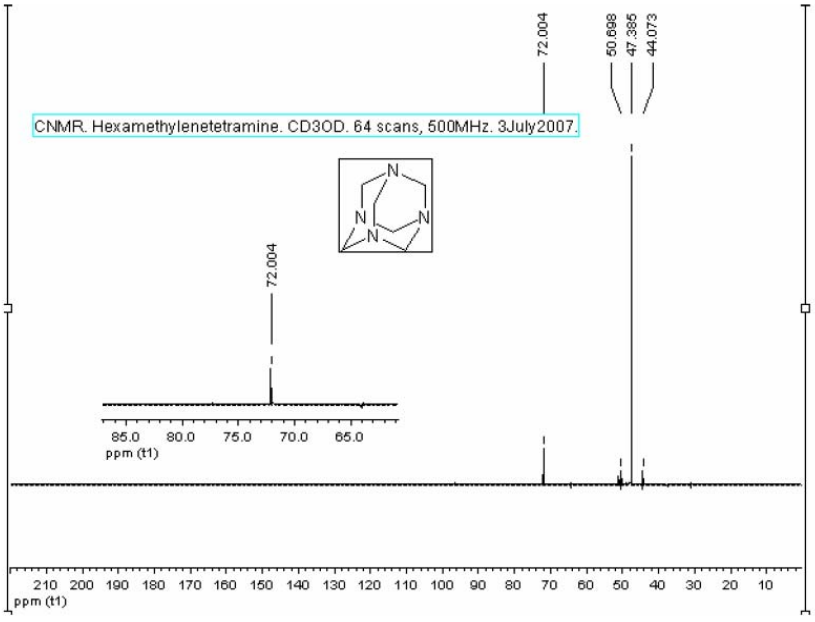


Figure 11 ^{13}C NMR of hexamine (UltaPure grade, Sigma-Aldrich)

Figure 12 A depicts the ^{13}C NMR spectrum of the RDX standard. It can be seen that the chemical shift of the RDX carbons is observed at 62.1 ppm

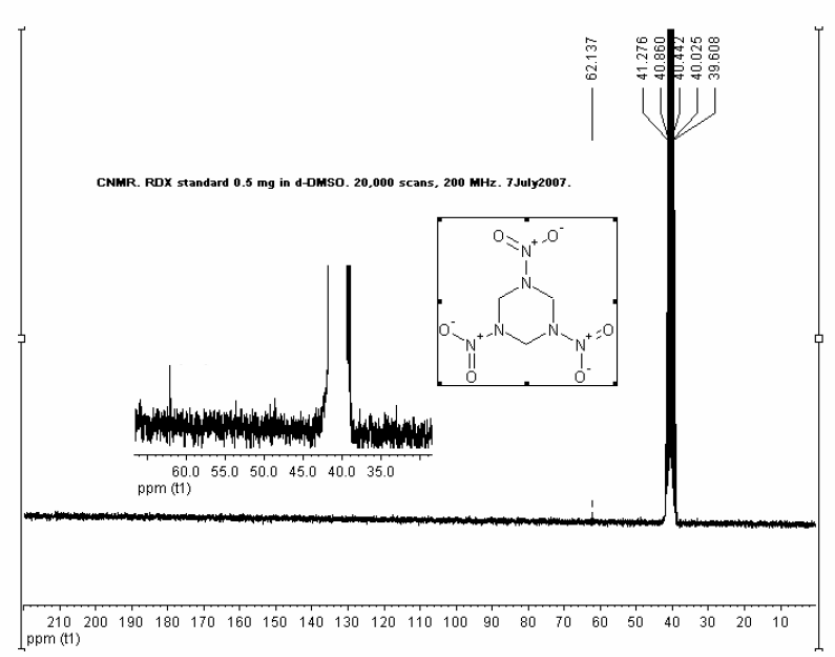


Figure 12A ^{13}C NMR spectrum of RDX standard (Accustandard, New Haven, CT)

The ^{13}C NMR spectrum of the reaction product showed the chemical shift of the carbon at 50.7 ppm (Figure 12B), which was very different from both hexamine and RDX.

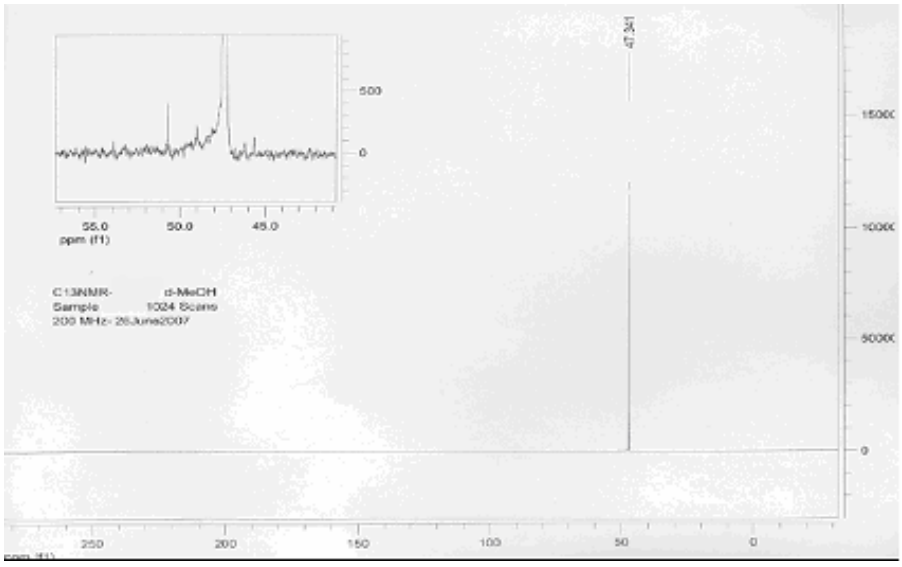
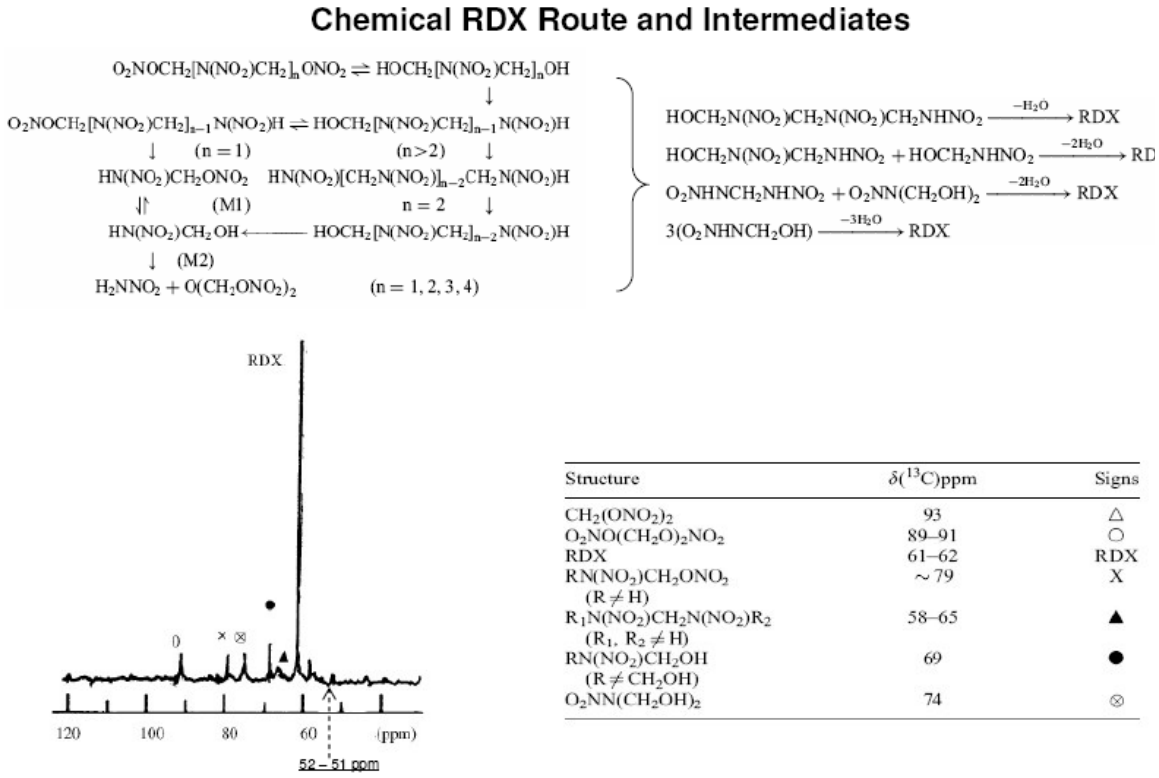


Figure 12B ^{13}C NMR spectrum of the bioconversion reaction product.

The comparison of the accumulated analytical data with the data on RDX and its intermediates available in the literature confirms that the biocatalytic product was not RDX as based on the ^{13}C signal assignments for RDX and its intermediates (5).

Figure 13 depicts the RDX chemical intermediates and their NMR assignment as referenced in the literature (5). It can be seen that the RDX ^{13}C NMR signal is located at 61-62 ppm, which is in line with 62.1 ppm shift observed by us in the RDX standard spectrum (Figure 12 A), and different from the 50.7 ppm shift detected in the spectrum of the biocatalitically produced product (Figure 12 B). However, among the small non-identified peaks of the RDX intermediates in Figure 13 a non-assigned peak at 52-51 ppm is also observed. This peak is similar to the 50.7 ppm peak in the spectrum of the bioconversion product (Figure 12 B). 1



Structure	$\delta(^{13}C)$ppm	Signs
$CH_2(ONO_2)_2$	93	△
$O_2NO(CH_2O)_2NO_2$	89–91	○
RDX	61–62	RDX
$RN(NO_2)CH_2ONO_2$ ($R \neq H$)	~79	X
$R_1N(NO_2)CH_2N(NO_2)R_2$ ($R_1, R_2 \neq H$)	58–65	▲
$RN(NO_2)CH_2OH$ ($R \neq CH_2OH$)	69	●
$O_2NN(CH_2OH)_2$	74	⊗

Figure 13 RDX chemical route intermediates (5)

6. CONCLUSIONS

In this Strategic Environmental Research and Development Program (SERDP), Infoscitex Corporation, University of Texas A&M, and the University of Massachusetts at Lowell evaluated possibility of biosynthesis of RDX from hexamine. The following conclusions were achieved:

- Multiple microbial strains deriving from Rhodococcus strain DN22 and exhibiting an improved Cytochrome P450 based enzymatic system for N-N bond cleavage in RDX can be successfully generated using error-prone PCR to create beneficial mutations. This system could potentially serve to create the N-N bond *de novo* in, for example, reaction of peroxynitrite with hexamine. These strains provide additional environmental benefits through their application for environmental clean up.
- The potential of microbial catalysis for bioconversion of hexamine into RDX was evaluated. The biocatalytic process resulted in formation of compound(s) that is different from RDX.
- The biocatalitically generated product was isolated, purified, and analyzed by FTIR and NMR. Based on the accumulated data, this compound may belong to a family of the RDX structural intermediates.

7. REFERENCES

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